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(54) Title: ALTERED STRAIN OF THE MODIFIED VACCINIA VIRUS ANKARA (MVA)

(57) Abstract: The invention provides new strains of the Modified Vaccinia Virus Ankara (MVA) that have a strongly reduced virulence for most mammals, especially humans, but nevertheless grows in cells of a continuous cell line approved for the production of a therapeutic agent such as a vaccine. The invention also provides a method for producing said adapted MVA strains. The adapted MVA can be used e.g. for parenteral immunization, as a vector system, or in the active or inactivated from as an adjuvant or as a regulator of the unspecific components of the immune system.

Altered Strain of the Modified Vaccinia Virus Ankara (MVA)

The present invention relates to new strains of the Modified Vaccinia virus Ankara (MVA) that have a strongly reduced virulence for most mammals, especially humans, but nevertheless grows in cells of a continuous cell line approved for the production of a therapeutic agent such as a vaccine. The invention also relates to a method for producing said adapted MVA strains. The MVA can be used e.g. for parenteral immunization, as a vector system, or in the active or inactivated form as an adjuvant or as a regulator of the unspecific components of the immune system.

Background of the invention

An organism is constantly challenged by infectious agents like bacteria, viruses, fungi or parasites. The immune system prevents the organism from permanent infection caused by these agents by the destruction and elimination of these infectious agents and any toxic molecules produced by them. The immune system can be divided into a specific and an unspecific part although both parts are closely cross linked. The unspecific immune response enables an immediate defense against a wide variety of foreign substances and infectious agents. In contrast, the specific immune response is raised after a lag phase, when the organism is challenged with a substance for the first time. However, the specific immune response is highly efficient. The specific immune response is responsible for the fact that an individual who recovers from a specific infection is protected against this specific infection but still susceptible for other infectious diseases. In general, a second infection with the same or a very similar infectious agent causes much milder symptoms or no symptoms at all. The immunity persists for a long time, in some cases even lifelong. This immunological memory is used for vaccination, where the organism is challenged with a harmless or inactivated form of the infectious agent to induce a specific

immunity. Sometimes adjuvants are incorporated into vaccines to enhance the specific immune response.

Much of the knowledge about infectious diseases and immunity is contributed by studies of smallpox. The disease is caused by the variola virus, a member of the genus of Orthopox viruses. Nearly two centuries ago, prophylactic inoculations with cowpox was initiated resulting in the immunization against smallpox. Later immunization was performed with the Vaccinia virus. In the early 1950s, many of the industrialized countries had eliminated endemic smallpox by using vaccination with Vaccinia virus. However, smallpox vaccination with this Vaccinia virus resulted occasionally in serious complications, such as postvaccinal encephalitis, generalized Vaccinia or contact infection.

A new vaccine that does not show these complications was developed by Anton Mayr. The pox vaccine consists of the pox virus Modified Vaccinia Virus Ankara (MVA) and was used for parenteral vaccination against smallpox in about 150 000 vaccinations without causing any complications related to the vaccination. Even children with immunologic deficiencies did not show serious side effects. The MVA was obtained by mutation and selection of the original vaccina virus Ankara after 575 passages in chicken embryo fibroblast cultures. The safety of this MVA is reflected by biological, chemical and physical characteristics. MVA has a reduced molecular weight, six deletions in the genome, and is highly attenuated for mammalian cells, i.e. DNA and protein is synthesized but virtually no viral particles are produced. The Modified Vaccina virus Ankara developed by Anton Mayr was deposited at the European Collection of Cell Cultures (ECACC), Salisbury, UK, under depository No. V 94012707.

The vaccination against smallpox was highly successful. In 1979, the World Health Organization declared the eradication of smallpox. Accordingly, the

mass vaccination of children was discontinued and only laboratory workers and members of the armed forces of some countries are vaccinated.

With the eradication of smallpox, the predominant cause of pox infection in humans was removed. However, some non-human poxviruses have reduced host specificity, i.e. they cause infections not only in their typical host (e.g. for cowpox the cow), but also in other animals, (e.g. rats and cats). Humans can be infected by this route as well. Since parts of the population are no longer immune against smallpox, orthopox infections of animal species can be dangerous for them. Domestic animals are the main source of infection for humans. Accordingly, the vaccination of domestic animals against orthopoxviruses is of increasing importance. In addition, the MVA may be of significance as a vector for gene therapy, i.e. to transfer nucleic acid sequences into a target cell where they are expressed.

For a logarithmic reproduction of the MVA, cell cultures of primary or secondary chicken embryo fibroblasts are needed. The cells are obtained from chicken eggs that are incubated for 10 to 12 days. Since eggs are subjected to a biological variability, the cells obtained for the cell culture system are variable on a cellular level as well. In addition, in a chicken embryo "fibroblast culture" often other cell types such as epithelial cells are found. This variation of the cells also results in variation of viruses produced in chicken embryo fibroblasts. It is therefore difficult to standardize and validate the cell culture system to guarantee a constantly high quality of the MVA produced. Furthermore, contamination of the cell culture system by microorganisms or viruses already present in the incubated eggs can not be completely excluded. When the MVA is grown in virus-contaminated cells, the MVA may recombine with the contaminating virus. Thereby an MVA with new and unpredictable characteristics may be generated. For the production of the virus in large scale in a suspension culture, primary or secondary chicken embryo fibroblasts are also not highly suitable. In addition, the purification and concentration of MVA by ultra

gradient centrifugation would be advantageous. However, such purification is difficult, when MVA is cultivated on primary or secondary chicken embryo fibroblast. Finally, an increasing number of patients have developed allergies against chicken egg's albumen. Although the *in vitro* conditions of the cultivation strongly reduce the allergenic potential, a hazard of an allergic reaction can not be completely excluded.

In conclusion, on the one hand the MVA can only be efficiently grown in primary or secondary chicken embryo fibroblasts causing a number of disadvantages, however, on the other hand the save application of the MVA in humans has been shown by its large-scale application as a vaccine.

Object of the invention

It is an object of the present invention to provide conditions for the production of homogeneous virus particles of the MVA. Additionally, said conditions should allow an easy and large-scale production of the MVA.

Detailed description of the invention

To achieve the foregoing and other objects, the present invention provides an MVA strain that is adapted for growing in cells of a continuous cell line, said cell line being approved for the production of a therapeutic agent.

According to the present invention, for the first time an efficient and large-scale production of MVA is possible. Since cells of a continuous cell line are homogeneous and their characteristics are stable the MVA harvested from these cell lines is also homogeneous with highly predictable characteristics. Furthermore, the risk of contamination by microorganisms can be controlled and contamination of the MVA preparation by proteins of the chicken egg - as found when cultivating MVA on chicken embryo

fibroblasts - can be excluded. The handling of a permanent cell line is convenient and thus highly suitable for industrial application.

In a preferred embodiment of the invention, the MVA is adapted for growing in cells of a mammalian cell line, which is approved for the production of a vaccine. It has been surprisingly found that the MVA adapted to a mammalian cell line such as the Vero cell line still has a reduced virulence for humans and also for a wide range of other mammals. Accordingly, the MVA is highly attenuated i.e. DNA and protein is synthesized but virtually no viral particles are produced, resulting in a virtually eliminated disease-causing capacity. Hence, the MVA according to the present invention is also highly suitable as a vaccine for humans and for a wide range of mammals. Accordingly, the MVA is especially applicable in the veterinary field.

Furthermore, a method to obtain an MVA strain according to the present invention is provided. According to this embodiment of the invention, cells of a cell line that is approved for the production of a therapeutic substance, are infected with the wild-type MVA. Preferably a high multiplicity of infection (MOI), i.e. a high number of viruses per cell is used for this infection. Then, the viruses are harvested and fresh cells of the same cell line are infected with the newly produced viruses. Said process is repeated (serial passaging) until the MVA is adapted to said cell line. Adaptation is reached, when 72h post infection, the virus titer is at least 1- to 9-fold, preferably 10- to 99-fold, more preferably 100- to 10^6-fold, and most preferably more than 10^7- to 10^{10}-fold increased compared to the input virus titer. The adaptation is reached after a limited number of passages.

"Adapted for growing" means that the amount of virus produced from an infection (Output) is increased compared to the amount of virus originally used to infect the cells (Input). In this case the Output/Input ratio is greater than 1.

"Derivative" of the MVA deposited at ECACC, Salisbury, UK, under the depository number 99101431 and/or provisional accession number 01021411 means an MVA which is adapted for growing in Vero cells at a rate, which is essentially the same as the growth rate of the deposited strain but carries at least one difference in its genome compared to the deposited strain.

The term "immune system" basically describes a complex involved in the defence of the organism against foreign substances and microorganisms. It is divided into a cellular part comprising several cell types, such as e.g. lymphocytes and other cells derived from white blood cells, and a humoral part comprising peptides and proteins such as antibodies, complement factors, and cytokins.

The term "immune response" describes the reaction of the immune system, when a foreign substance or microorganism enters the organism. Generally, the immune response is divided into a specific and an unspecific reaction although both are closely cross linked. The unspecific immune response is regarded as the immediate defence against a wide variety of foreign substances and infectious agents. The specific immune response can be characterised as a highly efficient defence mechanism of the organism against a foreign substance which is raised against said substance after a lag phase and highly specific for said substance. The specific immune response is responsible for the phenomenon that an individual who has recovered from a specific infection is protected against this specific infection in future.

"Activator of the immune system" means any substance capable of provoking or enhancing an immune response.

"Suppressor of the immune system" means any substance capable of reducing or inhibiting an immune response.

"Stabilizer of the immune system" means any substance capable of keeping the immune response on a constant level.

The inventors provide two preferred MVA strains that are adapted to an African green monkey cell line, called Vero cell line (ATCC No. CCL-81). The MVA-strain, which was passaged 100-times in Vero cells was called "Vero-MVA" and deposited at the European Collection of Cell Cultures, Salisbury, UK under depositary No. 99101431. The MVA strain after 200 passages in Vero cells was called "Vero-MVA-200" and deposited at ECACC under provisional accession number 01021411.

The MVA obtained as described above is further amplified by cultivating the cells of the approved cell line under suitable conditions, infecting cells with the MVA and harvesting the viral particles produced by said cells. Hence the MVA can efficiently and easily be amplified in large-scale. Surprisingly, the MVA of the invention does not show increased virulence in cells other than Vero cells such as human cell lines including HL, HEP-2 or HeLA.

In another embodiment of the invention, the MVA contains at least one heterologous nucleic acid sequence i.e. a nucleic acid sequence that is not naturally found in the MVA genome (recombinant MVA). Preferably, the heterologous nucleic acid sequence is a gene, more preferably a gene encoding an immunizing protein, and most preferably encoding a protein immunizing against malaria, rabies and/or hepatitis. The expression of said heterologous nucleic acid sequence is preferably under the transciptional control of a vaccinia virus promoter, more preferably of an MVA-own promoter. In a further preferred embodiment of the invention, the heterologous nucleic acid sequence is inserted at a naturally occurring deletion site in the MVA genome (disclosed in PCT/EP96/02926).

The recombinant MVA is used for the introduction of a nucleic acid sequence into a target cell, said nucleic acid sequence being homologous

or heterologous to the target cell. The introduction of a heterologous nucleic acid sequence into a target cell may be used to produce heterologous nucleic acids, peptides and/or polypeptides and/or proteins encoded by said nucleic acid sequence *in vitro*. This method comprises the infection of a host cell with the recombinant MVA, cultivation of the infected host cell under suitable conditions, and optionally isolation and/or enrichment of the peptide and/or protein produced by said host cell.

Furthermore, the introduction of a homologous or of a heterologous sequence may be applied for *in vitro* and preferably *in vivo* gene therapy. For *in vitro* and *ex vivo* gene therapy respectively, cells are isolated from the individual to be treated, transformed with the recombinant MVA and reintroduced into the individual the cells were taken from. For *in vivo* gene therapy, the recombinant MVA is directly administered to the living animal body including the human body. In a preferred embodiment of the invention, the recombinant MVA expresses an antigen or an antigenic epitope. Most preferably, said vector expresses an antigenic determinant from Plasmodium falciparum, Mycobacteria, Herpes virus, Influenza virus, hepatitis, or a human immunodeficiency virus.

Since the MVA according to the invention is – surprisingly – still highly attenuated, the MVA is ideal to immunize a wide range of mammals including humans. Hence, the present invention also provides a vaccine comprising the MVA for the immunization of a living animal body including a human against pox infections, preferably orthopox infections. The vaccine may contain in addition to the MVA one or more additives such as an antibiotic, a preservative, or a stabilizer. The vaccine is especially applicable in the veterinary field, e.g. for the immunization of animals against orthopox infections such as cats against cat pox, mice against ectromelia or camels against camelpox. The immunization is preferably performed parenterally.

The immunizing effect of an antigenic determinant in a vaccine is often enhanced by the addition of a so-called adjuvant. An adjuvant co-stimulates the immune system in an unspecific manner causing a stronger specific immune reaction against the antigenic determinant of the vaccine. According to another embodiment of the invention, the MVA is used as an adjuvant, to co-stimulate the immune response against the antigenic determinant of a vaccine. In this case it is preferred that the MVA is inactivated. The inactivation of the MVA may be performed e.g. by heat or chemicals. Preferably, the MVA is inactivated by β-propiolacton. According to this embodiment of the invention, the inactivated MVA may be added to vaccines against numerous infectious diseases to increase the immunity against this disease.

In case of an infection, the immune, the nervous, the hormonal and the vascular system of an individual work closely together. These interactions can be regulated by elements of the unspecific immune system e.g. cytokines such as interferons and interleukins. Pox viruses can influence the regulation of the immune system (Swiss Vet 11/99, 13-17). Hence, in a further embodiment of the invention, the MVA and preferably the inactivated MVA is used in mammals including humans to regulate the cellular and humoral elements of the unspecific (innate) immune system. Preferably the MVA is used as a bioregulator, wherein dysfunctions of the immune system are eliminated and the body's own defence mechanisms are activated, stabilized and/or suppressed. Most preferably, the MVA is used as a bioregulator in case of a viral infection e.g. with herpes, hepatitis B or C virus, in case of a chronic inflammatory disease and/or to support tumor therapy. The MVA may also be used to stabilize the immune system in a situation of increased susceptibility against infections such as in the case of stress or in neonatals. The active and/or preferably the inactivated MVA can be applied systemically e.g. intramuscularly and/or locally e.g. through mucous membranes and/or skin.

In conclusion, the present invention provides MVA strains that can in general be used for the same applications as the wild-type MVA, but eliminate the problems caused by the amplification of the wild-type MVA in chicken embryo fibroblasts.

Summary of the invention

The invention *inter alia* comprises the following, alone or in combination:

A modified vaccinia virus Ankara (MVA) adapted for growing in cells of a continuous cell line, said cell line being approved for the production of a therapeutic substance.

The MVA as above adapted for growing in cells of a mammalian cell line.

The MVA as above, wherein the cell line is approved for the production of a vaccine.

The MVA as above, wherein said approved cell line is a Vero cell line.

The MVA as above, wherein said approved cell line is the Vero cell line ATCC No. CCL-81.

The MVA as above, deposited at the European Collection of Cell Cultures, Salisbury, UK under depositary No. 99101431 and/or a derivative thereof.

The MVA as above, deposited at the ECACC, Salisbury, UK, under provisional accession number 01021411 and/or a derivative thereof.

The MVA as above, comprising at least one heterologous nucleic acid sequence.

The MVA as above comprising a heterologous nucleic acid sequence coding e.g. for a therapeutic protein and/or an antigenic determinant such as a peptide immunizing against malaria, hepatitis and/or rabies infection.

A host cell infected by the above described MVA.

A composition, preferably a pharmaceutical composition, comprising the above described MVA and/or the DNA of the MVA.

The pharmaceutical composition described above, wherein the pharmaceutical composition is a vaccine.

The vaccine described above for the immunization of a living animal body including a human.

The vaccine as above for the immunization against an Orthopox infection.

The vaccine as above for the immunization of cats against a cat pox infection, mice against ectromelia infection and/or camels against camelpox infection.

The pharmaceutical composition described above, wherein the MVA is an activator, suppressor and/or stabilizer of the unspecific immune system.

A pharmaceutical composition comprising the above described MVA and/or the DNA of the MVA as an adjuvant.

A pharmaceutical composition comprising the above described recombinant MVA and/or the DNA of the recombinant MVA.

The pharmaceutical composition as described above for use in gene therapy.

A method for introducing a homologous and/or heterologous nucleic acid sequence into a target cell comprising infection of the target cell with the above described MVA.

A method for obtaining an MVA strain as described above, comprising a) infection of cells of an approved cell line with a wild-type MVA, preferably the MVA deposited at ECACC under depository No. V 94012707, b) harvesting of the viruses, c) infection of fresh cells of the same cell line with the newly produced viruses, and, optionally, d) repetition of b) and c) until the virus is adapted to growth in cells of said cell line.

A method for producing viral particles of the above described MVA, comprising cultivating the cells of an approved cell line under suitable conditions, infecting said cell line with said MVA, and harvesting the viral particles produced by said cells.

The method as described above, wherein said cell line is infected with the MVA deposited at the ECACC under depositary No. 99101431 and/or the MVA deposited at the ECACC under provisional accession number 01021411 or a derivative of one of those strains.

A method for producing a nucleic acid sequence, a peptide polypeptide and/or protein, comprising infection of a host cell with the above described recombinant MVA, cultivation of the infected host cell under suitable conditions, and, optionally, isolation and/or enrichment of the nucleic acid sequence, peptide and/or protein produced by said host cell.

Use of the above described MVA for producing a pharmaceutical composition for the treatment or prevention of a disease or disorder responsive to said MVA

Use of the above described MVA for producing a vaccine for the immunization of a living animal body including a human.

Use of the above described MVA for producing an activator, suppressor and/or stabilizer of the unspecific immune system.

The use as described above for the manufacture of an adjuvant.

Use of the above described MVA as a vaccine.

Use of the above described MVA as an adjuvant.

Use of the above described MVA as an activator, suppressor and/or stabilizer of the unspecific immune system.

A method for immunization of a living animal body including a human said method comprising administering to a person in need thereof a therapeutically effective amount of an above described pharmaceutical composition.

A method for introducing a homologous and/or heterologous nucleic acid sequence into a target cell comprising infecting the target cell with the above described MVA and/or the DNA of the MVA.

A method for the activation, suppression and/or stabilization of the immune system of a living animal body including a human said method comprising administration of the above described pharmaceutical composition to a living animal body including a human.

A method for enhancing a specific immune response against an antigenic determinant in a vaccine comprising administration of the above described MVA as an adjuvant to a living animal body including a human.A Modified Vaccinia virus Ankara adapted for growing in cells of a continuous cell line obtainable by a process comprising the following steps:infecting cells of a cell line being approved for the production of a therapeutic substance, harvesting the viral particles produced by said cell lines and optionally, repeating the above steps until the desired growth characteristics of said MVA are obtained in said cells.

Examples

The following examples will further illustrate the present invention. It will be well understood by a person skilled in the art that the provided examples in

no way may be interpreted in a way that limits the applicability of the technology provided by the present invention to these examples.

Example 1: Adaptation of the MVA to Vero cells and characterization of said MVA strain

1. Adaptation of the MVA to Vero cells

The by Anton Mayr developed wild-type MVA that is a modified Vaccina virus Ankara was deposited at ECACC under depository No. V 94012707. The wild-type MVA was adapted to grow in Vero-cells by serial passaging of the virus in Vero cells (Table 1). The cell clone ATCC-No. CCL-81 of the stationary Vero cell line (WHO seed stock ECACC No. 88020401) was used in the passage No. 148 to 165 (WHO seed lot, Master and Working Bank). The cells were propagated in a medium consisting of Earle's MEM (ICN), pH 7,4 – 7,6, and 5% of the serum substitute BMS (Biochrom). According to a technique known by people skilled in the art, always the same cells of the working bank were seeded by splitting the cells 1:2 to 1:4. The medium contained approximately 250 000 cells per ml. The cells were respectively propagated in tubes (2ml), Roux dishes (100ml), and plastic dishes (6 and 40ml respectively). In general, the cells formed a confluent monolayer after 16 to 24h. Afterwards, the medium was replaced by plain Earle's MEM without any additives.

For the adaptation of the wild-type MVA a tube culture system was used. The results of the passages are summarized in Table 1 and 2. The Vero cells were infected by 10 MOI (multiplicity of infection) of the wild-type MVA, i.e. in average, 10 viral particles per Vero cell. The wild-type MVA to start with was a genetically homogeneous, plaque-purified MVA after 575 passages in chicken embryo fibroblasts (titer: $10^{7,75}$ KID_{50}/ml). After 24h, 90% of the Vero cells of the confluent monolayer were destroyed by toxic processes

(50% by toxicity, 40% by lysis). The medium plus the cell dedritus after freezing and thawing of the cells, containing the produced viruses, was harvested and 0,2ml of this mixture were seeded on the monolayer of Vero cells in the culture tubes (2nd passage). This procedure was repeated 200 times. After the third passage, no toxic effect was observed any more, whereas a mild cytopathic effect (CPE) characterized by rounding of the cells and lysis in a period of 4 to 6 days post infection (p.inf.) was seen. The virus titer was $10^{1,0}$ KID_{50}/ml. It was concluded that the proliferation of the MVA in Vero cells had started although very inefficiently. After the fifth passage, a typical CPE was observed which was completed after 4 to 5 days p.inf. The virus titer increased from $10^{1,0}$ KID_{50}/ml after the third passage to $10^{4,0}$ KID_{50}/ml after the fifth passage. Hence, the virus amplified more efficiently in Vero cells. In the passages No. 5 to 11, a complete CPE was observed more and more early and the virus titer increased with every passage. At passage No. 11, a plateau was reached at $10^{7,5}$ KID_{50}/ml. Accordingly, after eleven passages the adaptation of the MVA to Vero cells was achieved. In the following 30 additional passages, the results were for all passages the same and highly reproducible: The CPE began already 24h p.inf. and all cells were affected after three days p. inf. At that time, 20% of the Vero cells were rounded and 80% were lysed. After three days p.inf., the virus titer was always about $10^{7,75}$ KID_{50}/ml. After the fifteenth passage, the viruses were always harvested after two to three days p.inf., and only 1 MOI instead of 10 MOI were used to infect the cells (Table 2). In the following additional passages the growth characteristics of the MVA changed only slightly. Remarkably, the optimum virus titer further increased and reached 10^{10} KID_{50}/ml at passage 200.

In conclusion, the virus grows reproducibly in an exponential manner in Vero cells. Said growth characteristic is surprisingly different to the characteristics of the wild-type MVA. Accordingly, a new strain of the MVA was obtained by the serial passaging. Said new strain was called "Vero-MVA" and after passage 200 in vero cells "Vero-MVA-200".

The Vero-MVA and Vero-MVA-200 were cultivated in larger quantities. For storage, the Vero-MVA was concentrated by centrifugation, resuspended in 2,5 % polygeline and lyophilized in vials of 2ml. The titer after lyophilization was still at least $10^{8,5}$ KID_{50}/ml. The lyophilized Vero-MVA and Vero-MVA-200 was checked for contamination and toxicity and stored at +4°C.

2. Characterization of the biological properties of the Vero-MVA

The biological characteristics of the Vero-MVA (passage 100) and Vero-MVA-200 (passage 200) were compared with the characteristics of the wild-type MVA (Table 3 and Table 5). Thereby, the techniques known by the skilled practitioner were applied. The inventors showed that neither the host range of the virus was changed except for the Vero cells, nor the virulence for humans or animals was increased. The Vero-MVA is still characterized by the abortive propagation in non-permissive host cells.

The principal identity of the viral particles of the Vero-MVA compared to the viral particles of the Elstree strain of the Vaccinia virus was shown by cross reactivity of antibodies raised against the Elstree strain. The Elstree strain is a Vaccinia strain recommended by the WHO for the smallpox vaccination. The polyclonal hyperimmune serum of rabbits raised against the Elstree strain was added to the Vero-MVA. 100 KID_{50}/ml of the Vero MVA were completely neutralized at a dilution of the serum of 1:512. A twofold dilution of the serum was necessary to neutralize the same amount of Vaccinia Elstree strain (1:256). Accordingly, the Vero-MVA can still be efficiently neutralized by Vaccinia immune serum.

The Vero-MVA, the Vero-MVA-200 and the wild-type MVA were compared by a number of additional tests as indicated in Table 3, 4 and 5. The inventors showed that the virulence of Vero-MVA and Vero-MVA-200 for mammals including humans was not increased compared to the wild-type MVA. It was

also shown that the Vero-MVA and Vero-MVA-200 are not contagious or toxic for mammals including humans. Surprisingly, the cell specificity of the Vero-MVA was more or less identical to the specificity of the wild-type MVA except for the Vero cells: The Vero-MVA amplifies nearly as inefficiently in cells of human cell lines (see table 4: HL-, HEP-2-, and HeLa-cells) as the wild-type MVA does. Accordingly, although human cells and cells of African green monkeys are phylogenetically closely related, the Vero-MVA did not gain the ability to amplify in human cells. In other tests, no significant difference were seen either.

Furthermore, the physical, chemical, and biological characteristics of the wild-type MVA and the Vero-MVA-200 were compared (Table 5). Whereas the wild-type MVA growing in chicken embryo fibroblast cell cultures has three deletions in the left inverted terminal region, the Vero-MVA-200 has four deletions in the left terminal region compared to the genome of the pox virus as originally isolated in Ankara. Hence, passaging of the wild-type MVA in Vero cells resulted in an additional deletion.

The Vero-MVA was used to immunize domestic animals against Orthopox infections. The serum of the animals was collected and a neutralization test was performed. The inventors showed that the animals produced antibodies in high titers. The antibody titers were stable over a period of at least 111 days. It was also shown that the antibodies were able to neutralize *in vitro* viral particles of the MVA in a plaque-reduction test. In conclusion, the Vero-MVA can be used as a vaccine against Orthopox infections in domestic animals and in humans.

Table 1: Adaptation of the MVA to Vero cells

Passage No.	Cell culture	Highest virus titer [log_{10}/ml]	Result	Conclusion
1	toxic effect after 24h	2,0	Rests of the virus seeded	Blind passages Phenomenon of zones and cytokine production
3	No toxicity, moderate CPE after 4-6 days	1,0	Rests of the virus seeded? Begin of the virus reproduction	
5	typical CPE completed after 4-5 days	4,0	Increasing virus reproduction	
11	CPE completed after 3 days	7,5	Logarithmic virus reproduction	Adaptation successful
12-42*	CPE begins after 24h, completed after 3 days	7,75	Reproducible virus reproduction	Vero-MVA
43-100*	CPE begins after 24h, completed after 3 days	8,0	Repoducible virus reproduction	Vero MVA
100-200*	CPE begins after 24h, completed after 3 days	10,0	Repoducible virus reproduction	Results in Vero-MVA-200

* Only 1 MOI instead of 10 MOI are seeded after the eleventh passage.

Table 2: Change of the virus titers during the adaptation of the MVA to Vero cells

Passage No.	Harvested after [days p.inf.]	Titer per ml [log_{10}/ml]
1	1	<2,0
2	3	2,0
3	5	1,0
5	5	4,0
8	4	6,5
11	3	7,5
18	2	8,0
19	2	7,75
20	3	8,0
25	2	7,75
29	2	7,75
30	3	7,75
31	3	8,0
45	2	7,75
51	3	7,75
60	2	8,0
66	2	7,75
68	2	8,0
75	3	8,0
100	2	8,0
200	2	10,0

Table 3: Comparison of the biological characteristics of the wild-type MVA and Vero-MVA

Marker	Wild-type MVA	Vero-MVA (100. passage)	Vero-MVA-200
CPE in monolayer cell cultures (1 MOI seeded)	Rounding and lysis of the cells after day 5 (90% CPE)	Rounding and lysis of the cells after day 5 (100% CPE)	Rounding and lysis of the cells after day 3 to 5 (100% CPE)
Titer of the optimal harvest	$10^{8,0}$ KID_{50}/ml	$10^{7,75}$ KID_{50}/ml	$10^{10,0}$ KID_{50}/ml
Abortive virus reproduction in non-permissive cell systems	Yes	Yes	Yes
Reduced virulence for humans and animals	Yes	Yes	Yes: not virulent at all
Contagiousness	No	No	No
Character of the primary plaques on the chorion allantois membrane	No proliferative nodes without necrosis	No proliferative nodes without necrosis	No proliferative nodes without necrosis
Hemagglutination (chicken erythrocytes)	Negative	Negative	Negative
Inactivation by β-	Kinetic of first order for	Kinetic of first order for	Kinetic of first order for

propiolactone	0,05%	0,05%	0,04-0,05%
Protective effect in VSV-baby-mouse challenge test	Yes	Yes	Yes
Toxicity for humans and animals	No	No	No
Cytokine stimulation	Interferon α, IL-2, and 12, CSA	Interferon α, IL-2, and 12, CSA	Interferon α and γ, IL-1, 2, and 12, CSA
Activation of phagocytosis, natural killer cells, and T-lymphocytes	Yes	Yes	Yes, increased

Table 4: Reproduction rate in KID_{50}/ml of Vero-MVA and the wild-type MVA in different cell culture systems [log_{10}/ml]

Cell culture system	Vero-MVA (31.Vero-passage)	Wild-type MVA (575. passage in primary chicken embryo fibroblasts)
[1]) Vero (African green monkey kidney cells)	8,0	4,5
Primary chicken embryo fibroblasts	4,5	8,5
[1,2]) HL (human lung)	3,0	2,5
[1,2]) HEP-2 (human epidermoid carcinoma)	3,0	2,5
[1,2]) HeLA (human cervix carcinoma	2,75	2,75
[1,2]) BHK (hamster kidney cells)	5,75	5,25
[1,2]) MDBK (bovine kidney cells)	3,5	3,5
[1,2]) PK-15 (porcine kidney cells)	3,25	3,5

1) Continuous cell line derived from the tissue and species indicated in brackets.

2) Cell lines obtained from the collection of the institute of medical microbiology in Munich, Germany.

Table 5: Comparison of the wild-type MVA (572. passage in chicken embryo fibroblasts (CEF)) with Vero-MVA-200 (200. passage in vero cells)

Marker	Wild-type MVA	Vero-MVA-200
Genetic markers (comparison with pox virus strain as isolated in Ankara)	3 deletions in the left terminal region (inverted terminal repeat)	4 deletions in the left terminal region
	Genome size reduced from 208 to 178 kb	Further reduction of the genome size to 172kb
	Loss of 15% of the molecular weight of the original genome	Loss of 20% of the molecular weight of the original genome
	Loss of the interferon receptor	Additional loss of receptors e.g. for IL-1β
Cellular markers	Activation of T-helper cells (CD4, CD8, CD25)	Increased activation of cytotoxic T-lymphocytes
	Activation of NK cells	Increased activation of NK cells
	Abortive reproduction in mammalian cells (except BHK cells)	Further narrowing of the host spectrum in cell culture systems
Cytokine	Interferon α, IL-2, IL-12	Interferon α and γ, IL-1, 2, 12
Virus titer	CEF: $10^{9,5}$ KID_{50}/ml Vero cells: $10^{4,0}$ KID_{50}/ml	CEF: $10^{4,5}$ KID_{50}/ml Vero cells: $10^{9,5}$ KID_{50}/ml
Immune system	Reduction of activity of specific immune system	Inhanced activity of the unspecific immune system
Virulence for humans and animals	low	none

Applicant's or agent's file reference number	BN 33 PCT	International application No.	PCT/EP 01/02703

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, 7, 10, 12, 24, line 3, 10, 23, 16, 25.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution ECACC
Centre for Applied Microbiology and Research & European Collection of Cell Cultures

Address of depositary institution *(including postal code and country)*
Salisbury
Wiltshire SP4 OJG
United Kingdom

Date of deposit	Accession Number
February 15, 2001	(Provisional) 01021411

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g., EPC Rule 28(4); UK Patent Rules 1995, Schedule 2, Paragraph 3; Australian Regulation 3.25(3); Danish Patents Act Sections 22 and 33(3) and generally similar provisions mutatis mutandis for any other designated State.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*
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Applicant's or agent's file reference number	BN 03 PCT	International application No.	PCT/EP 01/02703

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, 7, 10, 12, 24, line 2, 8, 21, 14, 20.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution ECACC
Centre for Applied Microbiology and Research & European Collection of Cell Cultures

Address of depositary institution *(including postal code and country)*
Salisbury
Wiltshire SP4 OJG
United Kingdom

Date of deposit	Accession Number
October 14, 1999	99101431

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g., EPC Rule 28(4); UK Patent Rules 1995, Schedule 2, Paragraph 3; Australian Regulation 3.25(3); Danish Patents Act Sections 22 and 33(3) and generally similar provisions mutatis mutandis for any other designated State.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Authorized officer CLAUDIA ARAGONE	Authorized officer

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Claims

1. A modified vaccinia virus Ankara (MVA) adapted for growing in cells of a continuous cell line, said cell line being approved for the production of a therapeutic substance.

2. The MVA according to claim 1 adapted for growing in cells of a mammalian cell line.

3. The MVA according to claim 1 or 2, wherein the cell line is approved for the production of a vaccine.

4. The MVA according to any of the preceding claims 1 to 3, wherein said approved cell line is a Vero cell line.

5. The MVA according to claim 4, wherein said approved cell line is the Vero cell line ATCC No. CCL-81.

6. The MVA according to claim 5, deposited at the European Collection of Cell Cultures (ECACC), Salisbury, UK under depositary No. 99101431 and/or a derivative thereof.

7. The MVA according to claim 5, deposited at the European Collection of Cell Cultures (ECACC), Salisbury, UK under provisional accession number 01021411 and/or a derivative thereof.

8. The MVA according to any of the preceding claims 1 to 7, comprising at least one heterologous nucleic acid sequence.

9. The MVA according to claim 8 comprising a heterologous nucleic acid sequence e.g. a gene coding for a therapeutic protein and/or an antigenic determinant.

10. A host cell infected by an MVA virus according to any of the preceding claims 1 to 9.

11. A composition, preferably a pharmaceutical composition, comprising the MVA and/or the DNA of the MVA according to any of the preceding claims 1 to 9.

12. The pharmaceutical composition according to claim 11, wherein the pharmaceutical composition is a vaccine.

13. The composition according to claim 12 for the immunization of a living animal body including a human.

14. The composition according to claim 12 or 13 for the immunization against an Orthopox infection.

15. The composition according to claims 12 to 14 for the immunization of cats against a cat pox infection, mice against ectromelia infection and/or camels against camel pox infection.

16. The composition according to claim 11, wherein the MVA is an activator, suppressor and/or stabilizer of the unspecific immune system.

17. A composition, preferably a pharmaceutical composition, comprising the MVA and/or DNA of the MVA according to any of the preceding claims 1 to 9 as an adjuvant.

18. A composition, preferably a pharmaceutical composition, comprising the recombinant MVA and/or DNA of the recombinant MVA according to claim 8 or 9.

19. The composition according to claim 18 for use in gene therapy.

20. A method for introducing a homologous and/or heterologous nucleic acid sequence into a target cell comprising infection of the target cell with an MVA according to claim 8 or 9.

21. A method for obtaining an MVA strain according to any of the preceding claims 1 to 7, comprising

a) infection of cells of an approved cell line with a wild-type MVA, preferably the MVA deposited at ECACC under depository No. V 94012707,

b) harvesting of the viruses,

c) infection of fresh cells of the same cell line with the newly produced viruses, and optionally

d) repetition of b) and c) until the virus is adapted to growth in cells of said cell line.

22. A method for producing viral particles of the MVA according to any of the preceding claims 1 to 9, comprising

a) cultivating the cells of an approved cell line under suitable conditions,

b) infecting said cell line with said MVA, and

c) harvesting the viral particles produced by said cells.

23. The method according to claim 22, wherein said cell line is infected with the MVA according to claim 6 or 7.

24. A method for producing a nucleic acid sequence, peptide and/or polypeptide, comprising

a) infection of a host cell with the recombinant MVA according to claims 8 or 9,

b) cultivation of the infected host cell under suitable conditions, and optionally

c) isolation and/or enrichment of the nucleic acid sequence, peptide and/or protein produced by said host cell.

25. Use of the MVA according to any of the preceding claims 1 to 9 for producing a pharmaceutical composition for the treatment or prevention of a disease or disorder responsive to said MVA.

26. The use according to claim 25 for producing a vaccine for the immunization of a living animal body including a human.

27. The use according to claim 25 for producing an activator, suppressor and/or stabilizer of the unspecific immune system.

28. The use according to claim 25 for the manufacture of an adjuvant.

29. Use of the MVA according to claims 1 to 9 as a vaccine.

30. Use of the MVA according to claims 1 to 9 as an adjuvant.

31. Use of the MVA according to claims 1 to 9 as an activator, suppressor and/or stabilizer of the immune system.

32. A method for immunization of a living animal body including a human said method comprising administering to a person in need thereof a therapeutically effective amount of a composition according to claims 11 to 15.

33. A method for introducing a homologous and/or heterologous nucleic acid sequence into a target cell comprising infecting the target cell with the MVA and/or the DNA of the MVA according to claims 8 or 9.

34. A method for the activation, suppression and/or stabilization of the unspecific immune system of a living animal body including a human said method comprising administration of a pharmaceutical composition according to claim 16.

35. A method for enhancing a specific immune response against an antigenic determinant in a vaccine comprising administration of the MVA

according to any of the preceding claims 1 to 9 as an adjuvant to a living animal body including a human.

36. A Modified Vaccinia Virus Ankara (MVA) adapted for growing in cells of a continuous cell line obtainable by a process comprising the following steps:

a) infecting cells of a cell line being approved for the production of a therapeutic substance

b) harvesting the viral particles produced by said cell lines and optionally

c) repeating the above steps until the desired growth characteristics of said MVA are obtained in said cells.

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/EP 01/02703

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N7/08 C12N15/863 A61K39/275 A61K39/39 A61K48/00
A61K35/76 A61P31/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, MEDLINE, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 22978 A (MAYR ANTON) 31 August 1995 (1995-08-31) page 8, line 6 -page 9, line 14 ---	1-36
A	CARROLL MILES W ET AL: "Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: Propagation and generation of recombinant viruses in a nonhuman mammalian cell line." VIROLOGY, vol. 238, no. 2, 24 November 1997 (1997-11-24), pages 198-211, XP002174405 ISSN: 0042-6822 abstract --- -/--	1-36

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 2001	22/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Niemann, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DREXLER INGO ET AL: "Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells." JOURNAL OF GENERAL VIROLOGY, vol. 79, no. 2, February 1998 (1998-02), pages 347-352, XP002174406 ISSN: 0022-1317 abstract	1-36
A	WO 97 02355 A (GSF FORSCHUNGSZENTRUM UMWELT ;SUTTER GERD (DE); OHLMANN MARION (DE) 23 January 1997 (1997-01-23) cited in the application abstract	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-3,8-36 (partially searched)

Present claims 1-3,8-36 relate to a modified vaccinia virus Ankara (MVA) defined by reference to a desirable characteristic or property, namely growing in cells of a continuous cell line, said cell line being approved for the production of a therapeutic substance.

The claims cover all MVA having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such MVA. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the MVA adapted for growing in a Vero cell line 'see example 1 pages 14-17 !.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Inte nal Application No
PCT/EP 01/02703

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